DISTRIBUTION OF THE CYTOCHROMES OF SPINACH CHLOROPLASTS BETWEEN THE APPRESSED MEMBRANES OF GRANA STACKS AND STROMA-EXPOSED THYLAKOID REGIONS

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1. Introduction

The thylakoids of higher plant chloroplasts possess a *c*-type cytochrome, cyt *f* (E_m at pH 7.0, + 360 mV; λ_{max} of the α band; 554 nm) and three *b*-type cytochromes, cyt *b*-563 (E_m at pH 7.0, − 110 mV; λ_{max}, α band; 563 nm), cyt *b*-559$_{HP}$ (E_m at pH 7.0, + 370 mV; λ_{max}, α band; 559 nm) and cyt *b*-559$_{LP}$ (E_m at pH 7.0, + 20 mV; λ_{max}, α band; 559 nm) [1,2]. Cyt *f* and cyt *b*-563 are associated with photosystem (PS) I and cyt *b*-559$_{HP}$ with PS II [3]. Following the first separation of cyt *f* and cyt *b*-563 from PS I-enriched particles [4], a cyt *b*–*f* complex was isolated [5]. Now, an active intrinsic cyt *b*–*f* complex, a plastoquinone–plastocyanin oxidoreductase, has been isolated [6], which either mediates electron flow between PS II and PS I from plastoquinol to plastocyanin, or is involved in cyclic electron flow around PS I (see [7]).

The aim of this investigation was to determine the location of the various cytochromes and P700 along the membrane plane in chloroplasts containing grana, by comparing their distribution in subchloroplast fractions enriched in appressed or stroma-exposed membranes isolated by aqueous polymer two-phase partition [8]. Cyt *f*, cyt *b*-563 and cyt *b*-559$_{LP}$ are present in both the appressed membranes of the grana partitions and the stroma-exposed thylakoid regions. In contrast, cyt *b*-559$_{HP}$ is located mainly in grana partitions (the region where most of the PS II–LHCP complexes are located [8]), while P700, a marker for PS I complex, is located mainly in stroma-exposed thylakoids. Consequently, there is no evidence for a preferential location of the cyt *b*–*f* complex in either appressed membranes or stroma-exposed thylakoids; indeed it is nearly uniformly distributed between these 2 membrane regions. This contrasts with the heterogenous location of the other transmembrane intrinsic protein complexes, PS I complex, PS II complex, the light-harvesting complex and ATP synthetase (cf. [9]).

Abbreviations: chl, chlorophyll; cyt, cytochrome; PS, photosystem

2. Methods

Spinach thylakoids, stroma thylakoids (fraction Y-100) and vesicles derived mainly from grana partitions (fraction B3) were isolated by phase partition as in [8]. [Cyt] were determined by reduced minus oxidized difference spectra of fractions in 50 mM MES buffer (pH 6.0) [1]. [P700] were determined from ferricyanide-oxidized minus ascorbate-reduced difference spectra [10]. [Chl] were determined in 80% acetone as in [8]. A Perkin Elmer 557 spectrophotometer was used. Haem groups were stained with 3,3′,5,5′-tetramethylbenzidine–H_2O_2 [11,12], after resolution of the chl–proteins and haem–proteins on discontinuous SDS–PAGE slab gels (140 × 75 × 2.4 mm) with an 8–12% acrylamide gradient in the separating gel and 4% acrylamide stacking gel as in [13].

Cyt *f* was estimated from the hydroquinone-reduced minus ferricyanide-oxidized difference spectrum in 50 mM MES containing 1% Triton X-100 at pH 6.0, using an E_{mM} of 22 [1]. Cyt *f* and cyt *b*-559$_{HP}$ were determined from the hydroquinone-reduced minus ferricyanide-oxidized difference spectrum, using the simultaneous equations in [14]. Total cyto-

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chromes *b* (cyt *b*-559$_{HP}$ + cyt *b*-559$_{LP}$ + cyt *b*-563) were estimated from the dithionite-reduced minus ferricyanide-oxidized difference spectrum, and cyt *b*-559$_{LP}$ and cyt *b*-563 from the dithionite-reduced minus hydroquinone-reduced difference spectrum, according to [1]. Absorbance measurements were made at relevant peaks from a baseline drawn between 575 and 547 nm [2]; an E_{mM} of 20 was assumed for each of the cytochromes *b* [2].

3. Results

As the detergents used for thylakoid fractionation may release some cyt *f* and cyt *b*-563 [3], spinach thylakoids were mechanically fragmented by the Yeda press and stroma thylakoids (fraction Y-100) and grana stacks were isolated by differential centrifugation [8]. After fragmentation of the grana stacks by a further Yeda press treatment, an appressed membrane fraction B3, derived mainly from grana partitions, and highly enriched in PS II, was isolated by phase partition [14]. The comparison of the distribution of P700 (a marker for PS I complex) and cyt *f* (a marker for the cyt *b*–*f* complex) shown in table 1 confirms that the stroma-exposed membranes are enriched in P700, whereas the appressed membranes of grana partitions are highly depleted in P700, as shown in [15]. In contrast, the chl/cyt *f* molar ratios are rather similar in both fractions. The results show that the cyt *b*–*f* complex is present in both membrane regions, whereas PS I complex is found mainly in stroma-exposed thylakoids.

Comparison of the redox difference spectra (fig.1) of chloroplasts, and fractions B3 and Y-100 reveals that fraction Y-100 has little cyt *b*-559$_{HP}$, since the hydroquinone-reduced minus ferricyanide-oxidized difference spectrum has a 554 nm peak corresponding to cyt *f* only, and the dithionite-reduced minus ferricyanide-oxidized and dithionite-reduced minus hydroquinone-reduced difference spectra are rather similar. Fraction B3 is clearly enriched in cyt *b*-559$_{HP}$ compared to chloroplasts (fig.1). The average cytochrome concentrations (nmol/mg chl) of chloroplasts, B3 and Y-100 fractions, estimated from redox difference spectra, are compared in table 2. The concentrations of cyt *f* and of cyt *b*-563 are very similar, with molar ratios of cyt *f*:cyt *b*-563 of ~1:2 in all 3 cases. In contrast, cyt *b*-559$_{HP}$ is highly enriched in the grana partition fraction B3 (table 2). Values for cyt *b*-559$_{LP}$ are more difficult to estimate [1,2]. Nevertheless, the presence of cyt *b*-559$_{LP}$ in freshly prepared spinach thylakoids, and fractions B3 and Y-100, was shown both by the kinetic method [1] (table 1) and by the use of menadiol as a selective reductant of cyt *b*-559$_{LP}$ [2] (not shown). With the latter procedure [2], I found that cyt *b*-559$_{LP}$ occurred in freshly prepared spinach chloroplasts, confirming the results in [2] with lettuce chloroplasts. Although the function of cyt *b*-559$_{LP}$ is not known yet, it is a genuine thylakoid cyt and not a degradation product of cyt *b*-559$_{HP}$.

Fig.2 compares the chl–protein and haem–protein bands resolved by SDS–PAGE of chloroplasts and B3 and Y-100 fractions. Haem–polypeptides with app. M_r 33 000 and 18 000 were detected in each case. The 33 000 M_r band has been attributed

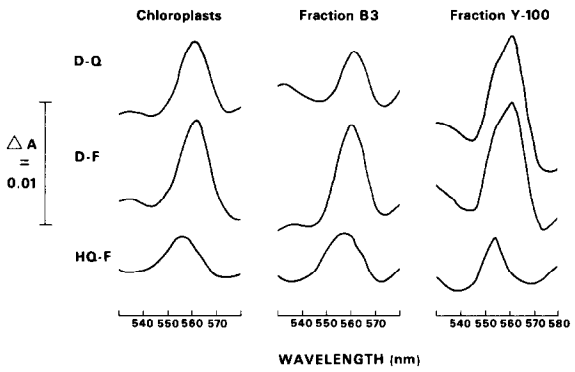

Fig.1. Redox difference spectra of spinach thylakoids (48 μg chl/ml), appressed membranes (B3 fraction) (40 μg chl/ml) and exposed membranes (Y-100 fraction) (80 μg chl/ml) in 50 mM MES, pH 6.0 (section 2).

Abbreviations: HQ/F, hydroquinone-reduced minus ferricyanide-oxidized; D/F, dithionite-reduced minus ferricyanide-oxidized; D/HQ, dithionite-reduced minus hydroquinone-reduced.

Table 1
Molar ratios of chl/P700 and chl/cyt *f* in chloroplasts, grana partitions and stroma-exposed thylakoids

Fraction	chl/P700[a]	chl/cyt *f*[a]
Chloroplasts	460	460
B3	1300	470
Y-100	220	485

[a] Values are averages of many determinations from 6 different preparations

Table 2
Comparison of cytochrome concentrations (nmol/mg chl)[a]

Fraction	cyt *f*	cyt *b*-563	cyt *b*-559$_{HP}$	cyt *b*-559$_{LP}$
Chloroplasts	1.6	3.2	3.1	1.5
B3	1.9	3.7	4.4	1.8
Y-100	1.9	3.7	0.3	1.4

[a] Values are averaged from several determinations with 6 different preparations of each fraction

to cyt *f* and the 18 000 M_r band to cyt *b*-563 [12]. In contrast to the very different relative proportions of the chl–proteins in appressed and stroma-exposed membranes (fig.2), the haem–polypeptides of both membrane fractions were qualitatively similar to those of chloroplasts. This confirms the above findings that the cyt *b*–*f* complex is uniformly distributed along the membrane plane in structurally differentiated thylakoids.

5. Discussion

5.1. *The location of the transmembrane supramolecular complexes*

The thylakoids of higher plant chloroplasts which have grana stacks possess 5 supramolecular transmembrane complexes, each containing several extrinsic and intrinsic polypeptides [16]. These are PS II complex, cyt *b*–*f* complex, PS I complex, chloroplast ATP synthetase and the light-harvesting chl *a*/*b*–protein complex. In structurally differentiated chloroplasts, there is a marked lateral asymmetry in the distribution of 4 of these complexes between the appressed membranes of grana partitions (whose outer membrane surface has only limited access to the stroma) and the stroma-exposed thylakoids. ATP synthetase is found exclusively in stroma-exposed thylakoids [17]. PS II complex and its associated light-harvesting complex are located mainly in the grana partitions and PS I complex is present mainly in stroma-exposed thylakoids [8]. Thus, while there is much heterogeneity in structure, composition and function along the chloroplast membrane, this study shows that the cyt *b*–*f* complex is rather uniformly distributed between appressed and stroma-exposed membranes in spinach thylakoids. Cyt *b*-559$_{HP}$, is known to be associated with PS II [3]. It is markedly enriched in grana partitions and depleted in stroma thylakoids (table 2) thus confirming not only that it

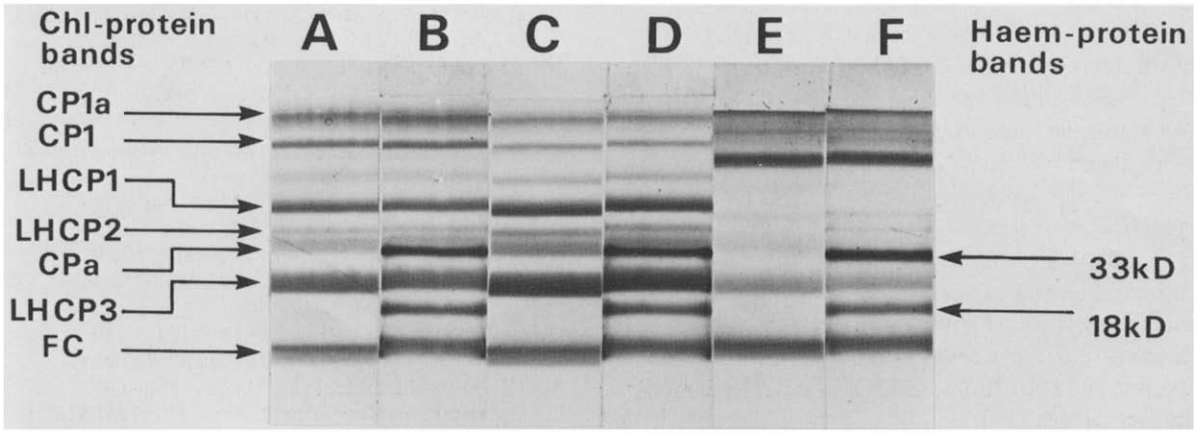

Fig.2. Chl–proteins of chloroplasts (A,B), fractions B3 (C,D) and Y-100 (E,F) resolved by SDS–PAGE slab gels [13]. CP1a and CP1 are chl–proteins associated with PS I complex, CPa with PS II complex, and LHCP$^{1-3}$ with the light-harvesting complex [13]. (B,D,F) were subsequently stained for haem groups [11,12] and had 2 haem–proteins with app. M_r 33 000 and 18 000.

is part of the PS II complex but also that PS II complex is associated mainly with appressed membranes [8].

5.2. *Conclusion*

Important consequences for electron transfer and coupled proton translocation in photosynthetic membranes arise from the finding that the cyt *b–f* complex is located in both appressed and stroma-exposed membranes, whereas the other complexes are preferentially located in one membrane region only.

(i) It demonstrates that the electron-transport components are arranged in supramolecular complexes which exist as discrete entities in the native membrane, rather than being fixed into single, structured electron-transport chains, as implied by the *Z* scheme. Although the compositions of individual complexes are probably constant, there need be no constant stoichiometry between the individual complexes themselves [17]. It is now recognized that P680/P700 molar ratios are rarely unity [18]. Further, much of the chl of PS II is structurally and stoichiometrically independent of that of PS I [8,9,15].

(ii) The uniform distribution of cyt *b–f* complex in contrast to the segregation of PS I and PS II complexes, suggests that the cyt *b–f* complex functions as a 2 electron carrier complex.

(iii) The presence of cyt *b–f* complex in both appressed and non-appressed membrane regions removes the need to postulate plastoquinone as the only mobile electron carrier linking PS II of the partitions with PS I in stroma-exposed thylakoids [8,9]. Both plastoquinone and plastocyanin are likely to be mobile electron carriers. Plastocyanin could either diffuse laterally while still attached to the inner membrane surface, or become detached from the membrane and diffuse in the intrathylakoid space which is continuous between grana partitions and stroma-exposed thylakoids. Plastocyanin would then have an analogous role to cyt *c* which links cytochrome reductase and cytochrome oxidase in inner mitochondrial membranes [19].

(iv) In [20] an alternative view of photosynthesis postulates that the two photosystems operate synchronously in parallel, rather than collaboratively in series as presumed in the *Z* scheme. In this scheme [20], $NADP^+$ and non-cyclic ATP are produced entirely by PS II activated by two quanta of light (the oxygenic photosystem); in parallel, the anoxygenic photosystem activated by one quantum of light (formerly called PS I) provides ATP by cyclic photophosphorylation. As pointed out [20], this scheme is consistent with the asymmetric distribution of PS II complex and PS I complex in different membrane regions [8]; my results suggest that the cyt *b–f* complex would need also to be involved in this postulated oxygenic photosystem.

(v) The presence of cyt *b–f* complex in both membrane regions, suggests that grana stacks will be the main site of non-cyclic photophosphorylation, with plastocyanin transferring electrons to those PS I complexes nearby in grana margins and end membranes, and thence to $NADP^+$ via ferredoxin–$NADP^+$ reductase, found only in stroma-exposed thylakoids [21]. Cyclic photophosphorylation involving the cyt *b–f* complex and PS I complex will be confined to stroma thylakoids.

References

[1] Bendall, D. S., Davenport, H. E. and Hill, R. (1971) Methods Enzymol. 23, 327–344.
[2] Rich, P. R. and Bendall, D. S. (1980) Biochim. Biophys. Acta 591, 153–161.
[3] Boardman, N. K. and Anderson, J. M. (1967) Biochim. Biophys. Acta 143, 187–203.
[4] Wessels, J. S. C. and Voorn, G. (1972) Proc. 2nd Int. Congr. Photosynth. Res. (Forti, G. et al. eds) pp. 833–845, Junk, The Hague.
[5] Nelson, N. and Neumann, J. (1972) J. Biol. Chem. 247, 1817–1824.
[6] Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599.
[7] Bouges-Bocquet, B. (1980) FEBS Lett. 117, 54–58.
[8] Andersson, B. and Anderson, J. M. (1980) Biochim. Biophys. Acta 593, 427–440.
[9] Anderson, J. M. (1981) FEBS Lett. 124, 1–10.
[10] Markwell, J. P., Thornber, J. P. and Skrdla, M. P. (1980) Biochim. Biophys. Acta 591, 391–399.
[11] Thomas, P. E., Ryan, D. and Levin, W. (1976) Analyt. Biochem. 75, 168–176.
[12] Høyer-Hansen, G. (1980) Carlsberg Res. Commun. 45, 167–176.
[13] Anderson, J. M. (1980) Biochim. Biophys. Acta 591, 113–126.

[14] Henningsen, K. W. and Boardman, N. K. (1973) Plant Physiol. 51, 1117–1126.
[15] Anderson, B., Åkerlund, H. E. and Albertsson, P. A. (1976) Biochim. Biophys. Acta 423, 122–132.
[16] Anderson, J. M. (1982) Photobiochem. Photobiophys. in press.
[17] Miller, K. R. and Staehelin, L. A. (1976) J. Cell Biol. 68, 30–47.
[18] Melis, A. and Brown, J. S. (1980) Proc. Natl. Acad. Sci. USA 77, 4712–4716.
[19] Hackenbrock, C. R. (1981) Trends Biochem. Sci. 7, 151–154.
[20] Arnon, D. I., Tsujimoto, H. Y. and Tang, G. M. S. (1981) Proc. Natl. Acad. Sci. USA 78, 2942–2946.
[21] Jennings, R. C., Garlaschi, F. M., Gerda, P. D. and Forti, G. (1979) Biochim. Biophys. Acta 456, 207–219.